REMARKS

Applicant respectfully requests reconsideration. Claims 143-147 were previously pending in this application. Claims 145-147 are amended. Claims 143-144 are canceled. New claims 148-150 are added herewith. Support for the new claim 148 is found in original claim 8. Claims 149 and 140 are dependent claims. As a result, claims 145-150 are pending for examination with claims 145 and 148 being independent claims. No new matter has been added.

Summary of Interview with Examiner

Applicant Martha Karen Newell and Helen Lockhart conducted a personal interview with Examiners VanderVegt and Saunders on July 12, 2007. Proposed arguments with respect to the rejection under 35 U.S.C. 112 were discussed and are provided in more detail below. Applicant very much appreciates the Examiners' willingness to discuss the arguments in the interview of July 12, 2007.

Rejection Under 35 U.S.C. 112

Claims 143-147 have been rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The basis for the rejection was discussed in the interview. In particular it was noted that the main issue in the rejection related to the scope of the terms ligand and HLA-DR antibody. It was asserted that the HLA-DR ligand is an agent that specifically is recognized by and binds into the antigen-binding groove of the HLA-DR molecule. Applicants disagree.

The HLA-DR ligand must bind to the HLA-DR molecule, but binding is not limited to the antigen binding groove of the HLA-DR. For instance the attached paper by Bertho et al (Human Immunology, 62: 106-112 (2001) describes studies involving HLA-DR mediated cell death. Cell death is induced using anti-HLA-DR monoclonal antibodies (see for instance Figure 4). The reference demonstrates that an HLA-DR specific antibody functions as an HLA-DR ligand to mediate cell death. Thus, an HLA-DR antibody is an HLA-DR ligand according to the claimed invention and does not necessarily need to bind to the antigen binding site.

Although Applicant disagrees with the rejection Applicant has canceled claims 143 and 144 and rewritten claims 145 in an independent form. Claims 146 and 147 are rewritten to depend from claim 145.

Applicant has also added new claims 148-150. Independent claim 148 is identical to original claims 8 and thus should not constitute new matter.

Further, Applicants wish to re-iterate that the instant claims were filed to copy the claims of US Patent No 6416958.

Considerations of Obviousness Type Double Patenting

Applicant calls to the Examiner's attention that the claims of US co-pending 11/027,053 have been allowed. If the Examiner believes that a Terminal Disclaimer is necessary in the instant application and the claims are otherwise in condition for allowance, he is requested to contact Applicant' s representative at the number listed below. Applicant is willing to consider filing a terminal disclaimer.

The claims of US 10/802,440 have also been allowed. However Applicant does not believe a terminal disclaimer is required over the claims of US 10/802,440. The parent application of US 10/802,440 was filed as a divisional application to cover the embodiments of Group III of the first restriction requirement of parent application US 09/277,575, (which was subsequently withdrawn) and Group VII –of the second restriction requirement of US 09/277,575. US 10/802,440 is a continuation of US 09/711,022 which is a divisional application of US 09/277,575. However, if the Examiner disagrees he is encouraged to contact Applicant' s representative at the number listed below.

The claims of US 09/277,575, have not yet been allowed to Applicant's knowledge. However if the claims have been allowed and it is believed that a Terminal disclaimer is required the Examiner is encouraged to contact Applicant's representative at the number listed below to discuss the potential filing of a terminal disclaimer.

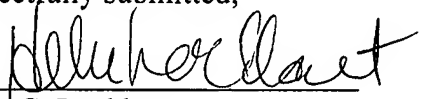
CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Dated: October 30, 2007

Respectfully submitted,

By 

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ELSEVIER

HLA-DR Mediated Cell Death is Associated With, but not Induced by TNF-α Secretion in APC

Nicolas Bertho, Béatrice Laupèze, Nuala Mooney, Claudine Le Berre, Dominique Charron, Bernard Drénou, and Renée Fauchet

ABSTRACT: Tumor necrosis factor α (TNFα) is a pleiotropic cytokine involved in inflammatory responses which can trigger both cell apoptosis and cell activation. In antigen presenting cells (APC), TNFα increased antigen presentation, notably by up-regulation of HLA class II expression. In addition to their role in antigen presentation, HLA-DR molecules transduce intracellular signals which lead to cytokine up-regulation or cell death. We have previously observed that the susceptibility of APC to HLA-DR mediated apoptosis increase throughout their maturation. We therefore investigated the relationship between TNFα production and susceptibility to HLA-DR-mediated apoptosis of different APC. The hematopoietic progenitor cell line (KG1), monocytic cell line (THP-1), monocyte-derived dendritic cell (DC), and B-lymphoid cell line (Raji) have been studied. We report that apoptosis susceptibility and spontaneous TNFα release are correlated in these different cells. However, while autocrine TNFα production was critical for DC maturation, upregulation of TNFα release after HLA-DR crosslinking was not observed and neutralization of endogenous TNFα did not modify HLA-DR-mediated apoptosis. These data reveal that HLA-DR mediated apoptosis susceptibility and spontaneous TNFα release are regulated in a parallel manner and that while TNFα may induce maturation of APC to an "apoptosis sensitive" stage, there is no direct role for TNFα in HLA-DR-mediated apoptosis of APC. *Human Immunology 62, 106–112 (2001).* © American Society for Histocompatibility and Immunogenetics, 2001. Published by Elsevier Science Inc.

KEYWORDS: tumor necrosis factor α; apoptosis; HLA-DR; antigen presenting cells; dendritic cells

ABBREVIATIONS

APC	antigen presenting cells
DC	dendritic cells
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA	human leukocyte antigen
IFNγ	interferon-γ
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
TCR	T-cell receptor
TNFα	tumor necrosis factor-α
TNF-R	tumor necrosis factor receptor

INTRODUCTION

Professional antigen presenting cells (APC) include B lymphocytes, monocytes, macrophages and dendritic cells (DC). APC present antigens from extracellular origin to T $CD4^+$ lymphocytes. Mature DC are considered the more potent APC and express a high level of HLA-DR molecules [1].

In addition to their role in antigen presentation, HLA class II molecules, and particularly HLA-DR, when they are crosslinked by TCR [2, 3], monoclonal antibodies (mAb) [4] or superantigens [5], have also the ability to transduce intracellular signal leading to cell activation [6], cell death [7] or cytokines release [8, 9], according to cell type and cell differentiation.

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CD34 positive hematopoietic progenitors, which express HLA-II molecules, are resistant to HLA-DR mediated cell death [10]. In the lymphoid lineage, the resting B cell does not enter apoptosis after HLA-DR crosslinking whereas the activated B cell does [7]. We have previously reported that HLA-DR mediated signals led to marked apoptosis in mature DC, significantly less in immature DC, and still less in macrophages, whereas monocytes were resistant to this cell death pathway [11]. Taken together, these data suggest a correlation between the presentation capacities of APC and the susceptibility to HLA-DR mediated cell death; leading to the notion that HLA-DR generated signals preferentially induce the demise of mature, activated professional APC.

Intracellular signals, generated by HLA-DR crosslinking also result in TNFα release in monocytes [5, 12] and in B lymphocytes. In B cells levels of released TNFα correlate with the stage of B-cell maturation, but do not correlate with the expression level of HLA-DR [13].

TNFα is an inflammatory cytokine that triggers apoptosis or activation according to the cell type and the receptor expressed (TNF-RI or TNF-RII, for review see [14]). However, in APC, TNFα is known as a cytokine that upregulates presentation capacities. This cytokine is involved in the upregulation of antigen presentation capacities of monocytes [15]. TNFα induces DC maturation, leading to upregulation of HLA-DR, CD80 and CD86, and consequently increases ability for antigen presentation (for review see [16]). TNFα also maintains B-cell proliferation [17] and facilitates induction of CD80 in human B cells [18].

Given the TNFα induced maturation APC on one hand and the sensitivity of mature APC to HLA-DR-mediated apoptosis, we have adressed the question of the role of TNFα production by APC in HLA-DR-mediated apoptosis. In three different APCs originating from myeloid and lymphoid lineages: activated B cells, monocytes, and dendritic cells have been compared with HLA-DR positive hematopoietic progenitor cells. The results lead us to suggest that autocrine production of TNF and sensitivity to HLA-DR mediated apoptosis are both regulated by maturation but that TNFα has no direct role in APC apoptosis.

METHODS AND MATERIALS

Reagents and Cytokines for Cell Cultures

The following mAb were used in cell cultures: monomorphic anti-HLA-DR mAb (L227 and L243) were purified from ascitic fluid. Monomorphic anti-TNFα mAb (mAb1), IgG1, and IgG2a isotype matched controls were purchased from Pharmingen (San Diego, CA, USA). All mAb used for cell cultures were conditioned without sodium-azide. GM-CSF, IL-4, TNFα, and IFNγ were purchased from TEBU (LePerray en Yvelines, France).

Cell Lines

Cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Raji is a B-cell line isolated from a Burkit Lymphoma. KG1 is a myeloid cell line ($CD34^+$, $HLA\text{-}DR^+$) isolated from an acute myeloid leukemia. THP-1 is a monocytic cell line isolated from an acute monocytic leukemia. All cell lines were cultured in RPMI supplemented with 10% FCS, 2 mM L-glutamine, and 1 mM pyruvate. THP-1 culture medium was supplemented with 2.10^{-5} M β_2-mercaptoethanol. For some experiments, THP-1 was cultured 48 h with 1000 U/ml IFNγ before assay.

Cell Purification and Generation of DC

After separation of peripheral blood mononuclear cells on Ficoll, cells were washed twice in HBSS (Life Technologies, Cergy Pontoise, France) with 10% fetal calf serum (FCS). They were resuspended at 3.10^6 cells/ml and incubated in 6-well microplates (3 ml/well). After a 2-h incubation at 37°C in 5% CO_2, nonadherent cells were removed. Adherent cells were cultured in IMDM (Life Technologies), 10% FCS supplemented with 800 U/ml GM-CSF, and 1000 U/ml IL-4. Half of the medium was replaced at days 3, 5, and 7 by fresh medium with 800 U/ml GM-CSF and 500 U/ml IL-4. At day 7, 100 U/ml TNFα was added to culture for 2 more days, to provide mature monocyte-derived DC.

Dosage of TNFα Present in Cell Culture

KG1, THP-1 cultured alone or with IFNγ, Raji and immature or mature DC were plated in 24-well plate at 5.10^5 cells/ml in IMDM, 10% FCS for 20 h in presence or absence of 5 μg/ml anti-HLA-DR mAb L243 or the relevant isotype matched control. Then cells were centrifuged and supernatant was collected for TNFα dosage. TNFα content of cell culture supernatant was measured using Immunotech (Marseille, France) TNFα ELISA kit, following manufacturer's instruction.

Culture of Raji and DC with Anti-TNFα or Anti-HLA-DR mAb

At the end of culture differentiation, immature or mature DC were plated in 24-well plate at 5.10^5 cells/ml in IMDM, 10% FCS for 20 h in presence or absence of 20 μg/ml anti-TNFα mAb, 5 μg/ml anti-HLA-DR mAb or the relevant isotype matched control. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Then cells were counted and divided for maturation and apoptotic analysis.

DC Maturation Characterization Using Flow Cytometry.

DC maturation was assessed by expression of CD83 on CD1a positive cells. Cells were incubated for 1 h in human AB serum, at 4°C, to avoid nonspecific mAb binding. The following mAb were used for immunolabeling: FITC- or phycoerythrin-conjugated (PE-conjugated) mouse mAb against CD1a and FITC labeled isotype controls were purchased from Immunotech, whereas mAb against CD83 and PE-labeled isotype controls were purchased from Pharmingen. After immunolabeling, cells were analyzed by flow cytometry using a Cytoron flow cytometer (Ortho, Raritan, NJ, USA) equipped with an argon laser operating at 488 nm. Data were acquired with the Immunocount II software (Ortho).

Detection of Cell Death

For studies of induced apoptosis cells were incubated for 1 h in human AB serum at 4°C, before staining with PE-labeled anti-CD34, -CD33, -CD1a, or -CD19 mAb for, respectively, KG1, THP-1, DC, or Raji cell line. Then cells were incubated with FITC-labeled Annexin-V (Pharmingen) to determine apoptosis. Flow cytometry gating allowed us to select cells that exhibit CD34, CD33, CD1a, or CD19 antigens, and to analyze their binding of Annexin-V, which is a membrane marker of apoptotic cells [19].

Specific apoptosis was calculated as follow: % specific apoptosis = 100 × [(% of Annexin-V + cell in assay) − (% of Annexin-V + cell in control)]/[100 − (% of Annexin-V + cell in control)].

Statistics

The comparison between variables was analyzed using the Student's *t*-test.

RESULTS

Differential Susceptibility to HLA-DR Mediated Cell Death of Cell From Different Origins

First, we have investigated susceptibility to HLA-DR mediated cell death of the different cells used (Figure 1). As we have previously described [11], KG1 and THP-1 do not undergo apoptosis after HLA-DR crosslinking (respectively 0.1% ± 0.4% and 0.1% ± 0.5%). Incubation of KG-1 and THP-1 with IFNγ induces HLA-DR upregulation (data not shown), but although THP-1 become weakly, but significantly, sensitive to HLA-DR-mediated apoptosis (2.3% ± 1.2%), KG-1 remain insensitive (data not shown). Immature DC died a bit more than THP-1 plus IFNγ (4.5% ± 1.7%), whereas mature DC are highly sensitive to HLA-DR mediated cell death (31.8% ± 11.4%), as are Raji (48.3% ± 12.9%).

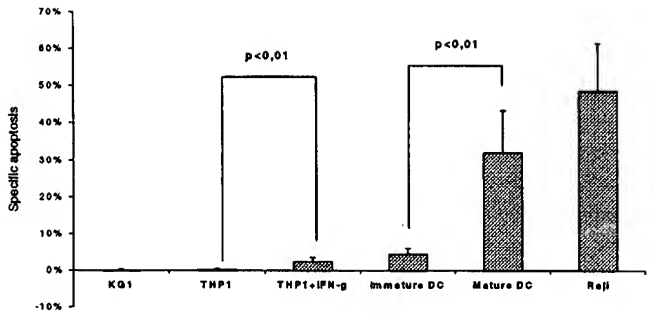

FIGURE 1 Sensitivity of different HLA-DR expressing cells to HLA-DR mediated apoptosis. Cells were cultured at 5.10^5 cells/ml, in presence of 5 μg/ml anti-HLA-DR, L243, or isotypic control (IgG2a). After the 20-h culture, cells were stained with PE-labeled anti-CD34, -CD33, -CD1A, or -CD19 mAbs for, respectively, KG1, THP-1, DC, or Raji cell line. Then cells were incubated with FITC-labeled Annexin-V in order to determine apoptosis using flow cytometer. THP-1 + IFNγ was cultured 48 h with 1000 U/ml IFNγ before assay. Data represent mean ± SD of four samples, *p* was calculated by Student's *t*-test.

Differential TNFα Secretion of the HLA-DR Positive Cell Lines

We studied TNFα release by these different cell types, during a 20-h culture (Table 1). Neither KG1 nor THP-1 secrete detectable levels of TNFα. However incubation of THP-1 with IFNγ induce a significant although weak TNFα release (63 ± 23 pg/ml). Immature DC secrete an equivalent amount of this cytokine with 56 ± 12 pg/ml whereas maturation of DC upregulates TNF release (130 ± 16 pg/ml). Raji cells produce an equivalent amount of TNFα (145 ± 42 pg/ml) to mature DC (Table 1).

The comparison between HLA-DR mediated apoptosis susceptibility (Figure 1) and the spontaneous TNFα

TABLE 1 Spontaneous TNF-α release of different HLA-DR positive cell lineages

	KG1	THP-1	THP-1 + IFN γ	Immature DC	Mature DC	Raji
IgG2a	0 ± 0	35 ± 30	63 ± 23	56 ± 12	130 ± 16	145 ± 42
L243	1 ± 1	29 ± 25	69 ± 24	64 ± 30	140 ± 14	153 ± 38

Mean ± SD of TNFα released in cell culture supernatant (pg/ml) during a 20-h culture.

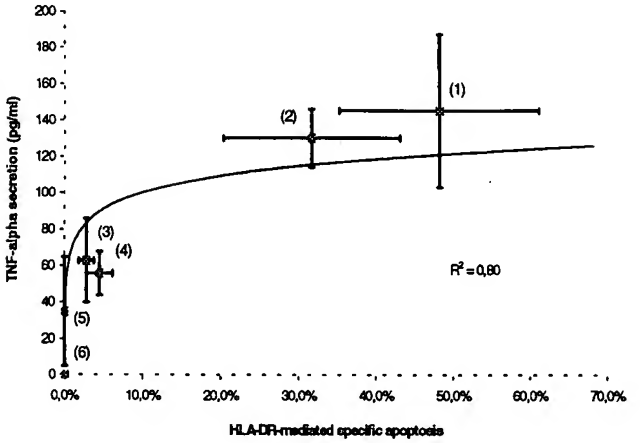

FIGURE 2 Correlation between susceptibility to HLA-DR mediated apoptosis and spontaneous TNFα secretion. Results depicted in Figure 1 and Table 1 have been compiled to assess correlation between these data: (1) Raji, lymphoblastoid cell line; (2) mature DC, derived from normal monocytes; (3) immature DC, derived from normal monocytes; (4) THP-1 + IFNγ, monocytic cell line; (5) THP-1, monocytic cell line; and (6) KG1, myeloid cell line $CD34^+$ (isolated from AML). R^2 = correlation coefficient for a logarithmic curve.

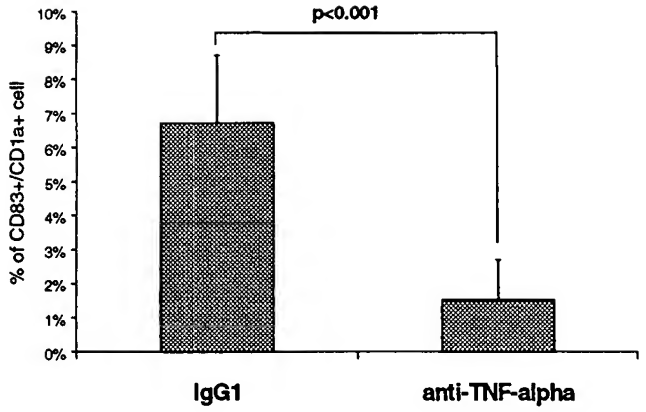

FIGURE 3 Neutralization of endogenous TNFα inhibits spontaneous DC maturation. Immature DC have been incubated in presence of 20 μg/ml anti-TNFα mAb or matched isotype control (IgG1). After a 20-h incubation, DC were stained with anti-CD83-FITC and CD1a-PE mAb, and then analyzed using flow cytometry. The histogram depict mean ± SD of four samples, p was calculated by Student's t-test.

release (Table 1) highlights a correlation between amount of TNFα in the supernatant and HLA-DR-mediated apoptosis of the cells (Figure 2).

Spontaneous TNFα Secretion of DC Induce Their Maturation

The maturation state of immature DC was studied to demonstrate that TNFα released is biologically active and that anti-TNFα mAb is able to neutralize endogenous TNFα. We used CD83-FITC staining, of immature DC cultured with or without neutralizing anti-TNFα mAb. As shown in Figure 3, a small proportion of immature DC undergo maturation spontaneously (6.7% ± 2.0 $CD83^+$ cells), whereas the same cells, cultured in the presence of an anti-TNFα mAb, fail to undergo maturation (1.5% ± 1.2% of $CD83^+$ cells). These data therefore demonstrate that TNFα released by DC is biologically active.

Endogenous TNFα Does not Affect Neither DC nor Raji Survival

Because TNFα induces apoptosis of some cell types, we investigated the influence of spontaneous TNFα release on cell survival. Immature and mature DC survival in presence or not of anti-HLA-DR mAb was not affected by neutralization of endogenous TNFα. In 20-h culture, in presence of L243, immature DC present 10.3% ± 2.5% of apoptotic Annexin-V+/$CD1a^+$ cells, which is unaltered from the level detected in cells cultured in the presence of anti-HLA-DR and anti-TNFα mAb: 9.6% ± 2.5% (Figure 4). The percentage of mature DC death was 47.4% ± 11.2% whereas, in the presence of anti-TNFα MoAb, the percentage of apoptotic cells was 55.7% ± 12.8% (Figure 4a). Raji cell line present the same unalteration of HLA-DR mediated apoptosis with or without neutralizing anti-TNFα mAb with, respectively, 47.4% ± 4.0% and 50.6% ± 3.0% (Figure 4b).

DISCUSSION

HLA-DR-mediated apoptosis susceptibility of APC is correlated with the antigen presentation capacity of APC [11] and TNFα upregulates antigen presentation in monocytes [15], DC [16], and B lymphocytes [18]. The relationship between HLA-DR mediated apoptosis and TNFα release has not previously been investigated. The aim of this study was to investigate the role of TNFα release in HLA-DR-mediated apoptosis.

An increase in TNFα mRNA and in TNFα protein level after HLA-DR cross linking, in monocytes [5, 9], similar to B lymphocytes, has been described [13]. We did not observe increased TNFα secretion using the described protocol although the biologic activity of the TNFα was confirmed by its capacity to increase CD83 expression on DC.

We observed increasing susceptibility to HLA-DR mediated apoptosis of the $CD34^+$ cell line KG1, the monocytic cell line THP-1, the monocyte-derived immature and mature DC, and the lymphoid cell line Raji in agreement with our previous data [11]. Analysis of spontaneous TNFα release in these different cell lineages highlights a correlation between TNFα release and HLA-DR mediated apoptosis susceptibility. KG1 and

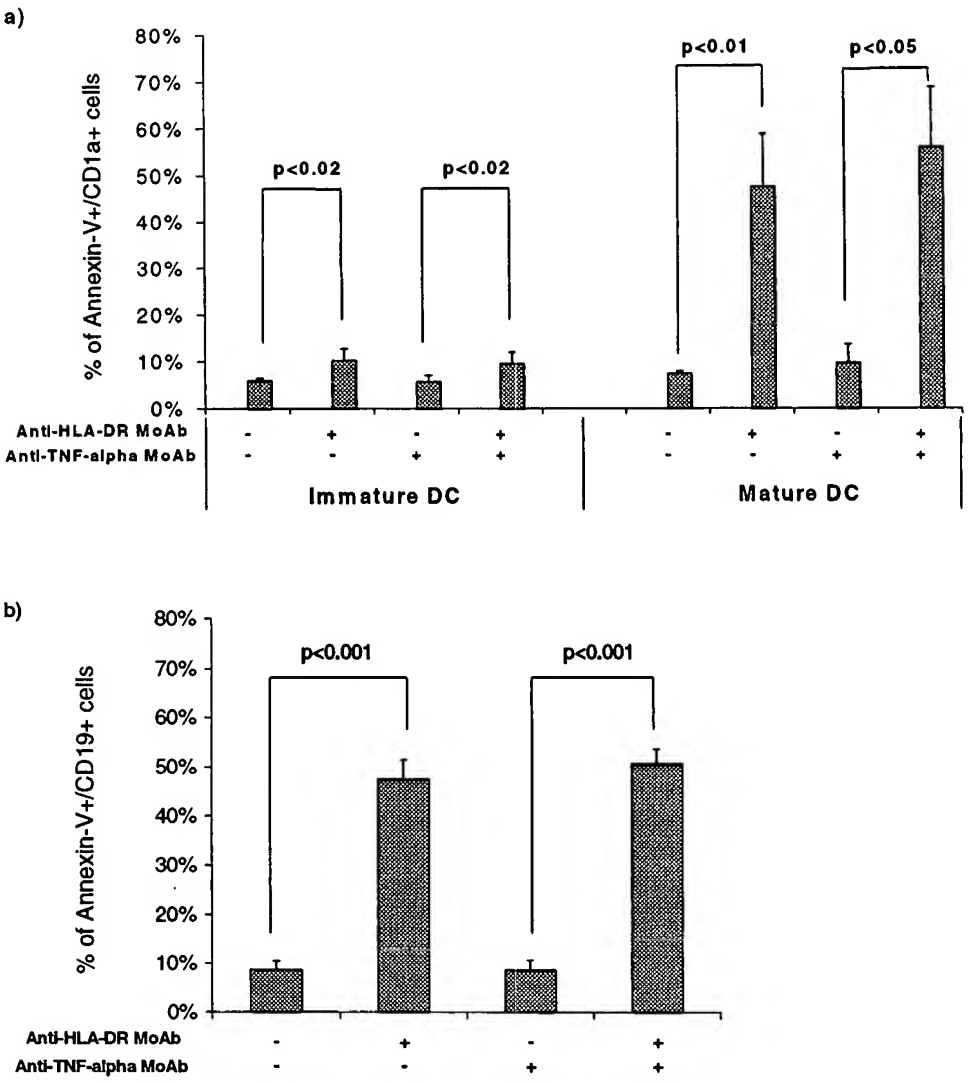

FIGURE 4 Neutralization of endogenous TNFα does not modify HLA-DR mediated apoptosis of DC and Raji. Raji and immature or mature DC have been incubated in the presence of 20 μg/ml neutralizing anti-TNFα mAb and/or 5 μg/ml anti-HLA-DR mAb or their respective isotype control. Percentage of apoptotic cells was assessed the same way as shown in Figure 1. The histogram depict mean ± SD of four samples, p was calculated by Student's t-test.

THP-1, which do not undergo apoptosis, do not secrete TNFα; THP-1 plus IFNγ and immature DC, which undergo little apoptosis, secrete little TNFα; and mature DC and Raji cell line produce a lot of TNFα and are highly sensitive to apoptosis after HLA-DR signaling. The observed upregulation of TNFα release during IFNγ activation of monocytes, and during maturation of DC have been previously described [20, 21]. We examined the potential relationship between HLA-DR mediated apoptosis and spontaneous TNFα release, which could be envisaged in two ways.

First, a direct role of TNFα in HLA-DR-mediated apoptosis; this would be possible if HLA-DR-signaling induced significant upregulation of TNFα release, but this was not the case in our experimental conditions. In B cells, it has been demonstrated that HLA-DR mediated cell death is not mediated by a secreted factor [22]. DC express cFLIP [23], an intracellular protein that interacts with FADD and/or caspase-8 and inhibits Fas and TNFR1-induced apoptosis [24]. We have previously demonstrated that HLA-DR mediated cell death is caspase independent in B cells [22] and in mature DC [11]. However, TNFα could also be implicated in HLA-DR mediated cell death if HLA-DR signaling modifies sensitivity of APC to TNFα, as has been observed for Fas [25]. However, HLA-DR crosslinking did not modify FLIP expression in DC (data not shown). Finally, the addition of a neutralizing anti-TNFα mAb did not interfere with HLA-DR mediated cell death, which definitively excludes a direct role of TNFα in HLA-DR mediated cell death.

Second, a common pathway involving both the increase of TNFα release during APC differentiation/maturation, and the installation of HLA-DR mediated apoptosis signaling mechanism could be envisaged. TNFα expression is regulated by two pathways: the well known regulation of the transcription and the less known regulation of the mRNA stability [26]. Numerous tran-

scription factors like Egr-1, c-Jun [27], and AP-1 [28] are able to regulate TNFα expression, but the main factors implicated in TNFα regulation belong to NF-κB/Rel family [29], a transcription factors family involved in the regulation of immune responses [30]. Among these proteins, RelB is particularly interesting because its nuclear localization increase during DC differentiation and B-cell activation [31], in parallel to the susceptibility to HLA-DR mediated cell death, CD80 and CD86 expression [11], and TNFα release (Figure 2). Moreover mice knock out for RelB present chronic inflammation and enlarged lymph nodes in agreement with an important role of this protein in the immune homeostasis [32].

We have previously demonstrated that HLA-DR mediated cell death is a common feature of all mature APC and that this apoptosis can occur independently of Fas/Fas-L interaction. We now demonstrate that TNFα, despite is increasing expression during APC differentiation/maturation, is not directly implicated in HLA-DR mediated apoptosis.

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